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Fusions to the cholera toxin B subunit: influence on pentamerization and GM1 binding

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Abstract

The cholera toxin B (CTB) subunit has been used extensively in vaccine research as a carrier for peptide immunogens due to its immunopotentiating properties, where coupling has been obtained either by genetic fusion or chemical conjugation. For genetically fused immunogens both N- and C-terminal fusions have been used. Only shorter extensions have previously been evaluated and in some reports these fusions have impaired the biological functions of CTB, such as the ability to form pentamers and to adhere to its cell receptor, the GM1 ganglioside. Here we report the first systematic study where the same fusion partner has been used for either C-terminal, N-terminal or dual fusions to CTB. The serum albumin binding region (BB, approximately 25 kDa) from streptococcal protein G, which is known to fold independently of N- or C-terminal fusions, was selected as fusion partner. The three fusion proteins CTB-BB, BB-CTB and BB-CTB-BB were expressed in *Escherichia coli*, where they were efficiently secreted to the periplasmic space, and could be purified by affinity chromatography on human serum albumin (HSA) columns. The CTB fusion proteins were compared for their ability to form pentamers, by gel electrophoresis and size-exclusion chromatography, and it was concluded that all three fusion proteins were able to pentamerize. Interestingly, the C-terminal fusion to CTB showed most efficient pentamerization, while the dual fusion was much less efficient. Purified pentamer fractions from all three fusions where found to react to a monoclonal antibody described to react only to pentameric forms of CTB. In addition, the purified pentamer fractions were analyzed in an enzyme-linked immunosorbent assay (ELISA) for their ability to bind GM1, and it was found that the C-terminal fusion (CTB-BB) showed significant GM1-binding, but that also the N-terminal and dual CTB fusion proteins bound GM1, although less efficiently. The implications of the results for the design and use of CTB fusion proteins as subunit vaccines are discussed. © 1997 Elsevier Science B.V.

Keywords: Cholera toxin B subunit; Fusion protein; Streptococcal protein G; Pentamerization; GM1 binding

Abbreviations: CT, cholera toxin; CTB, cholera toxin B subunit; GM1, GM1 ganglioside; HSA, human serum albumin; LTB, *E. coli* heat-labile enterotoxin B subunit; PBS, phosphate buffered saline (pH 7.4); PBST, PBS containing 0.05% Tween 20; SpA, *Staphylococcus aureus* protein A; SpG, streptococcal protein G

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1. Introduction

Cholera toxin (CT) consists of a non-covalently assembled pentamer of the non-toxic B subunit (CTB), responsible for cell attachment, and a toxicogenic A subunit, post-translationally cleaved into the A1 and A2 peptides (Mekalanos et al., 1983). CTB consists of 103 amino acids and has a molecular mass of 11.6 kDa. In the holotoxin, CTB is, in a pentameric form (Hardy et al., 1988), responsible for the binding to the GM1 ganglioside, which is present on all nucleated mammalian cells and abundant on intestinal epithelial cells. Both CT and CTB have been extensively studied due to their importance in the virulence of *Vibrio cholerae*, but also for their impact as immunopotentiators upon delivery of heterologous antigens in vaccine development (McGhee et al., 1992; Holmgren et al., 1993, 1994; Nashar et al., 1993). Although CTB sometimes is referred to as being a mucosal adjuvant (Tamura et al., 1988; McKenzie and Halsey, 1984; Chen and Strober, 1990; Oien et al., 1994), a more generally accepted opinion today is that the true adjuvant activity can be attributed to the A subunit or contaminating amounts of the cholera holotoxin in such cases when non-recombinant CTB is used for immunizations (Czerkinsky et al., 1989; Holmgren et al., 1994; Staats et al., 1994).

When using CTB as a carrier molecule for foreign antigenic determinants, mucosal route immunizations have in numerous reports resulted in high serum IgG antibody titers as well as secretory IgA in mucosal secretions (Hirabayashi et al., 1990; Nashar et al., 1993; Lehner et al., 1994). Protective immunity to respiratory syncytial virus in mice was recently reported using this strategy (Oien et al., 1994). CTB is thus of utmost interest for mucosal vaccine development, and its immunopotentiating capacity as a carrier molecule is considered to be related to its ability to bind to the monosialoganglioside GM1 (Dertzbaugh and Elson, 1993b; Holmgren et al., 1993; Staats et al., 1994).

In certain cases when chemical conjugation of the foreign antigen to CTB has been employed (Nashar et al., 1993), the CTB-conjugates have failed to evoke the expected immune responses (McGhee et al., 1992), and it has been discussed whether this is due to the fact that chemical coupling procedures could affect the immunogenicity by chemical modifications of the target immunogen or by generating a heterogeneous population of CTB-conjugates, where only a fraction retains the ability to form pentamers which can bind to GM1 (McGhee et al., 1992). Recombinant strategies, where a gene fragment encoding the target immunogen is fused to the CTB-encoding gene, for subsequent expression in a heterologous host such as *Escherichia coli*, offer the advantages of CT-free, and thus non-toxic preparations as well as favoring more uniform immunogen compositions (Staats et al., 1994).

Fusion proteins carrying shorter extensions to CTB in either the N-terminus (Sanchez et al., 1988, 1990; Dertzbaugh et al., 1990; Dertzbaugh and Elson, 1993a,b; González et al., 1993) or the C-terminus (Sanchez et al., 1990; Cheng-hua et al., 1995; Zhang et al., 1995a,b), or even internally within the CTB sequence (Bäckström et al., 1994, 1995), have been produced and characterized. The results from these reports, and reports from studies of similar fusions to the structurally closely related *E. coli* heat-labile enterotoxin B subunit (LTB) (Schödel et al., 1990; Lipscombe et al., 1991), concerning retained functionality of the CTB- or LTB-fusion proteins, respectively, are inconclusive. While González et al. (1993) demonstrated that a CTB fusion protein carrying an 18 amino acid N-terminal extension showed retained activity with respect to pentamerization and GM1 binding, Dertzbaugh and Elson (1993b) found that short N-terminal extensions, being 8, 12 or 24 amino acids in length, respectively, induced structural changes that impaired the GM1 binding capacity of these CTB-fusions. Sandkvist et al. (1987) demonstrated that rather small modifications in the N-terminus of LTB could dramatically decrease pentamerization ability and thus GM1 binding. While Jagusztyn-Krynicka et al. (1993) showed that large proteins (> 100 kDa) could be fused to the C-terminus of LTB with retained ability to bind GM1, Clements (1990) showed that a C-terminal fusion to LTB inhibited pentamer formation.

Up to now no systematic study has been performed to determine if N- or C-terminal fusions to larger protein domains could affect the ability of CTB fusions to pentamerize, an ability which has been shown to be required for GM1 binding (Hardy et al., 1988). We have therefore designed and pro-

duced three different CTB fusion proteins that are characterized in this study. The gene encoding an albumin binding region (BB) (Nygren et al., 1988) from streptococcal protein G, has been fused either C-terminally, N-terminally or at both termini of the CTB-encoding gene. BB, being 25 kDa in size, has been used extensively as an albumin-binding affinity tag (Nygren et al., 1994) and is known to fold independently of fused target proteins, both when introduced as an N-terminal or C-terminal fusion partner (Hammarberg et al., 1989; Öberg et al., 1993), respectively. The three generated fusion proteins, CTB-BB, BB-CTB and BB-CTB-BB, which could be efficiently recovered by affinity chromatography from the *E. coli* periplasm, have been characterized in this comparative study for their ability to form pentamers. Furthermore, formed pentamers, assembled from the three different CTB fusion proteins, have been purified by size-exclusion chromatography and compared for their ability to bind the GM1 ganglioside.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains RRIΔM15 (Rüther, 1982) and KS476 (Strauch and Beckwith, 1988, 1989) were used as bacterial hosts for subcloning and protein expression, respectively. Plasmids pRIT28 (Hultman et al., 1988), pEZZ18 (Löwenadler et al., 1987) and pE318 (Nguyen et al., 1995) were used for subcloning. Plasmid pJS162 (Sanchez and Holmgren, 1989) was used as template in the polymerase chain reaction (PCR), for the amplification of the CTB-encoding fragment, and pB1B2mp18 (Ståhl et al., 1989) and pRIT24 (Hammarberg et al., 1989) were used as sources for the two variants of BB-encoding gene fragments.

2.2. DNA-constructions

DNA-construction work was performed essentially as described by Sambrook et al. (1989). The CTB gene was amplified by PCR, performed according to a standard protocol (Hultman et al., 1989), with the CTB specific primers CTB3 (5′-GGG-GAATTCCACACCTCAAAATATTACTGAT-3′) and CTB4 (5′-CCCCAAGCTTAGTCGACATT-TGCCATACTAATTGC-3′), containing restriction enzyme recognition sites in non-complementary sequences for further cloning. The plasmid pJS162, encoding the 569B CTB, was used as template in the PCR. The obtained gene fragment encodes the complete CTB, except the signal peptide. The PCR gene fragment was restricted with *Eco*RI and *Hin*dIII and ligated into plasmid pRIT28, previously digested with the same enzymes, to generate the plasmid pRIT28CTB2. The nucleotide sequence was verified by solid phase sequencing (Hultman et al., 1991).

Plasmid pB1B2mp18 encodes the serum albumin binding region, denoted BB (Nygren et al., 1988), of streptococcal protein G (SpG), preceded by the staphylococcal protein A (SpA) signal sequence. The plasmid pRIT24 encodes a dual affinity fusion protein consisting of two IgG binding domains, ZZ (Nilsson et al., 1987), derived from SpA, followed by the BB region from SpG. A multicloning site for insertion of genes is situated downstream the BB-gene and the ZZ-gene in the two vectors, respectively. The CTB gene fragment was isolated from pRIT28CTB2 with either *Eco*RI/*Hin*dIII or *Eco*RI/*Sal*I restriction and inserted into pB1B2mp18 cleaved with *Eco*RI and *Hin*dIII and pRIT24 cleaved with *Eco*RI and *Sal*I, respectively. The obtained expression vectors, denoted pEBBCTB and pRIT24CTB, encode the fusion proteins BB-CTB and ZZ-CTB-BB, respectively. Plasmid pRIT24CTB was restricted with *Eco*RI and *Hin*dIII, to obtain a gene fragment encoding CTB-BB, which was isolated and inserted into the plasmids pB1B2mp18 and pE318, previously restricted with the same enzymes. Plasmid pE318, containing the SpA signal sequence followed by a multicloning site, is derived from plasmid pEZZ18. Plasmid pE318 was obtained by deletion of the ZZ gene fragment through restriction with enzyme *Acc*I, as the ZZ gene is flanked by *Acc*I sites, followed by religation of the plasmid. Expression plasmids pEBBCTBBB and pECTBBB encode the fusion proteins BB-CTB-BB and CTB-BB, respectively. Gene expression and fusion protein secretion to the periplasmic space from all the expression plasmids constructed, are under control of the SpA transcription and secretion signals.

2.3. Antibodies

The monoclonal antibody LT39, specifically reactive with CTB pentamers (González et al., 1993), and used in the Western blot analysis, was kindly provided by Professor Holmgren. The polyclonal anti-CTB rabbit serum used in the GM1 ELISA was obtained by immunizing rabbits with commercial CTB (Sigma) in Freund's complete adjuvant according to standard procedures.

2.4. Production of CTB-fusions

Tryptic Soy Broth (500 ml, Difco, Detroit, MI) containing Yeast Extract (5 g/l, Difco) and ampicillin (100 mg/l, Sigma, St. Louis, MO) was inoculated with *E. coli* KS476 transformed with either pECTBBB, pEBBCTB or pEBBCTBBB. The cells were grown over night at 30°C. The overnight cultures were harvested by centrifugation at 3000 *g*. Periplasmic fractions were isolated by an osmotic shock procedure (Nossal and Heppel, 1966). Recombinant fusion proteins were purified by affinity chromatography on human serum albumin (HSA) Sepharose as described by Ståhl et al. (1989). Relevant fractions, as determined by absorbance at 280 nm, were pooled and lyophilized.

2.5. Protein characterization

2.5.1. SDS–PAGE analysis

Affinity purified fusion proteins were analyzed under non-reducing conditions in SDS–PAGE loading buffer (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2.5% SDS and 0.01% bromphenol blue) or reducing conditions (SDS–PAGE loading buffer containing 5% β-mercaptoethanol (Laemmli, 1970) on SDS–PAGE, using either 8–25% gradient gels, on the Pharmacia Phast system (Pharmacia Biotech, Uppsala) or on the BioRad Mini-Protean II system (BioRad, Hercules, CA), using 12% homogenous gels, according to the suppliers' recommendations. The protein bands were visualized with Coomassie blue R250 staining. The expression levels for the three fusion proteins in shake-flask cultivations ranged from 6–12 mg per liter of culture.

2.5.2. Size-exclusion chromatography

Both analytical and preparative size-exclusion chromatography were performed using a Superose™ 12 PC 3.2/30 column and the Pharmacia SMART™ system (Pharmacia Biotech, Uppsala). The CTB fusion proteins (1 μg/μl) were incubated over night at 37°C in 0.1 M Tris–HCl, pH 7.5, 0.4 M NaCl and 2 mM EDTA, in order to allow pentamerization. Ten or 20 μg of protein was loaded on to the column, previously equilibrated with phosphate buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, pH 7.4), for the analytical and preparative gel filtration, respectively. Molecular mass standards (LMW and HMW gel filtration calibration kits, Pharmacia Biotech) were separated under the same conditions to allow size estimations of collected fractions. Protein detection was performed by absorbance measurement at 280 nm, and relevant fractions were pooled for further characterizations.

2.5.3. Pentamerization analysis

The fractions from the size-exclusion chromatography expected to contain pentamers of the CTB fusion proteins were analyzed by Western blotting. One μl of the pentamer fractions, containing approximately 100 ng of fusion protein, was mixed with 2 μl non-reducing SDS–PAGE loading buffer, immediately before analysis on SDS–PAGE using the Mini-Protean II System, as described above. The protein bands were transferred to nitrocellulose filters by electroblotting, followed by blocking of the filters in PBS containing 1% milk solids at room temperature for one hour. After three washing steps in PBS, containing 0.05% Tween 20 (PBST), CTB-containing protein bands were detected by incubation at room temperature for one hour with a pentamer-specific monoclonal antibody LT39. Visualization of bound monoclonal antibodies was achieved by incubation with alkaline phosphatase conjugated goat–anti-mouse antibodies (Sigma) in PBST, at room temperature for one hour, followed by three washing steps in PBST and development in 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma *FAST*™, Sigma) according to the suppliers' recommendations. Pre-stained marker standards (SeeBlue™, Novex, San Diego, CA) were used to allow size estimations of blotted proteins.

2.5.4. GM1 ELISA

The pentamer fractions from the size-exclusion chromatography were further analyzed for GM1

binding activity in a GM1-ganglioside enzyme-linked immunosorbent assay (GM1 ELISA) essentially as described by Svennerholm et al. (1986). Briefly, polystyrene microtiter plates (Nunc, Roskilde) were coated with GM1 monosialoganglioside (Sigma), 1 μg/ml, diluted in 10 mM PBS, pH 7.4 (100 μl per well) at 37°C for two hours, followed by three washing steps with 10 mM PBS. The remaining binding sites were blocked with 200 μl/well of 0.5% gelatin (Biorad EIA reagent, Biorad) in PBS for one hour at room temperature. The pooled pentamer fractions, obtained by the preparative size-exclusion chromatography as described above, were serially diluted in PBS, and added to the wells, 100 μl per well, and incubated at room temperature for 1.5 h. After binding of proteins, the wells were washed three times as described above and incubated with 100 μl CTB-reactive rabbit serum diluted 1:1000 in PBS, containing 0.05% gelatin, and the plates were incubated at room temperature for one hour. After washing, the plates were incubated with 100 μl horse radish peroxidase-conjugated goat–anti-rabbit IgG (Dako, Glostrup), diluted 1:2000 in PBS with 0.05% gelatin, and the plates were incubated at room temperature for one hour. Hundred μl TMB (3,3′,5,5′-tetramethylbenzidine) microwell peroxidase substrate (Dako) was added to the wells after a washing step, followed by a ten minutes incubation at room temperature. The reaction was terminated by addition of 100 μl 1 M H_2SO_4 per well, followed by an absorbance measurement at 450 nm. An affinity purified irrelevant BB fusion protein, BB-M3 (Ståhl et al., 1990) produced as described above for the CTB fusion proteins, and commercial pentameric CTB (Sigma), were used as a negative and positive controls, respectively, in the GM1 ELISA. Additional negative controls consisted of wells to which either no GM1, or no rabbit anti-CTB IgG was added (data not shown).

3. Results

3.1. Design of the CTB fusion proteins

In order to evaluate whether CTB could be fused to larger protein regions with still retained ability of CTB to form pentamers and to bind GM1, and to investigate if the N- or C-terminus of CTB would be more suitable for fusion, three different CTB fusion proteins were designed. The albumin binding region BB (Nygren et al., 1988), from streptococcal protein G, was genetically fused C-terminally, N-terminally or at both termini of CTB. Three different fusion proteins were thus generated: CTB-BB (36.7 kDa), BB-CTB (37.7 kDa) and BB-CTB-BB (61.5 kDa). The BB region was chosen as fusion partner because it has the ability to fold independently of fused proteins (Hammarberg et al., 1989; Öberg et al., 1993), and it has a size (approximately 25 kDa) which is significantly larger than the previously reported peptide extensions of CTB, and finally for the reason of simplified recovery. Due to the albumin binding capacity, the produced fusion proteins can be efficiently purified by human serum albumin (HSA) affinity chromatography.

3.2. Expression and characterization of the gene products

The three different CTB fusion proteins expressed in *E. coli* were directed to the periplasmic space by the secretion signals of staphylococcal protein A (SpA) (Ståhl et al., 1989). After osmotic release of the periplasmic content, the three fusion proteins were affinity purified on HSA–Sepharose (Ståhl et al., 1989). SDS–PAGE analysis under reducing conditions showed expected sizes for the purified fusion proteins (Fig. 1A). The full-length products were the dominating bands although some degradation products were visible. These smaller sized bands are most probably due to a certain degree of proteolytic degradation, but they should originate from the fusion proteins since they could be affinity purified on HSA. Under non-reducing conditions, internal disulfide bonds can be formed within the CTB subunits, and CTB-pentamers can assemble (Hardy et al., 1988). The non-covalent interactions responsible for pentamer formation are strong enough for analysis of intact CTB-pentamers by non-reducing SDS–PAGE (Hardy et al., 1988). To evaluate the possibility of pentamer formation of the fused CTB variants, the three affinity purified fusion proteins were incubated under conditions expected to favor pentamerization, and thereafter subjected to SDS–PAGE analysis under non-reducing conditions (Fig. 1B). The

Coomassie stained gel shows bands of various sizes which presumably indicate multimerization, but the multimerization is not complete since the bands which correspond to the monomeric forms are still present. The sizes of the full-length bands of the monomers correspond well with expected sizes (Fig. 1B, arrowheads). The sizes for the larger bands would correspond well to pentameric forms of the CTB-BB and BB-CTB fusion proteins (Fig. 1B, lanes 1 and 2) with the molecular weights of 183 kDa and 188 kDa, respectively. The sizes of the multimeric forms of BB-CTB-BB exceed the separation range of the SDS–PAGE gel and is thus only seen as a band between the stack gel and the separation gel (Fig. 1B, lane 3). The pentamer of the dual fusion protein has a calculated molecular weight of 307 kDa. A similar analysis of non-fused BB demonstrates that BB has no ability to form multimers (data not shown). It is not possible from this experiment to firmly conclude that pentamers are formed, nor is it possible to estimate the ratio between monomeric and multimeric forms of the fusion proteins. Nevertheless, all three CTB fusion proteins demonstrate multimerization ability, and it is tempting to speculate that pentamers exist for all the CTB-fusions, among perhaps also other forms. Size-exclusion chromatography was thus employed to enable further characterization.

3.3. Analysis of pentamerization ability of the CTB fusions

The affinity purified CTB fusion proteins, incubated under conditions expected to favor pentamerization, were analyzed by size-exclusion chromatography (Fig. 2). The chromatograms for the three fusion proteins showed a similar pattern; after the void-peak which is displayed on the left (retention time 8 min), a peak appears (peak I) which might correspond to pentameric forms of the respective CTB fusion proteins. A second peak (peak II) probably represents monomeric forms of the CTB fusion proteins (Fig. 2). Pentameric forms are thus seen for

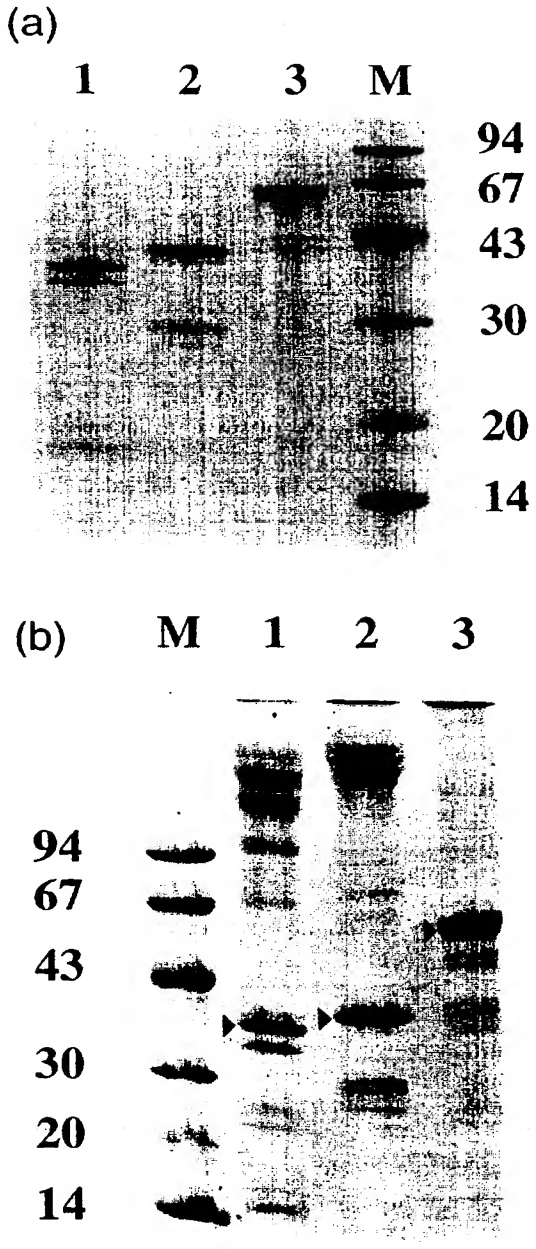

Fig. 1. SDS–PAGE analyses of the CTB fusion proteins. The expressed and affinity purified CTB fusion proteins were subjected to SDS–PAGE analyses: (A) 12% homogeneous gel under reducing conditions and (B) 8–25% gradient gel under non-reducing conditions after incubation favoring pentamerization. Lane 1, CTB-BB; lane 2, BB-CTB; lane 3, BB-CTB-BB; lanes M, marker proteins with molecular masses in kilodaltons. Approximately 3 μg of fusion protein was loaded in each lane. Full-length bands of the monomeric fusion proteins are marked with arrowheads in (B).

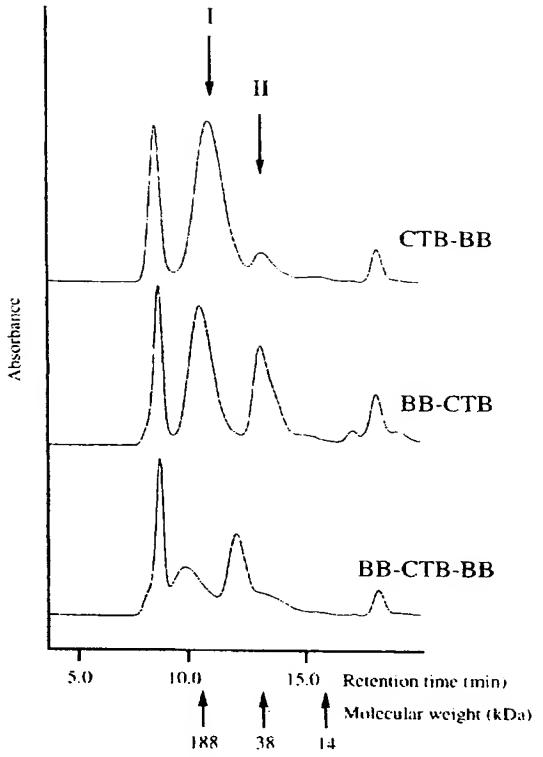

Fig. 2. Size-exclusion chromatography analyses of the CTB fusion proteins. Peaks I and II have retention times which suggest that they should correspond to pentameric (peak I) and monomeric (peak II) forms of the three CTB fusion proteins, respectively. The molecular weight markers are based on gel filtration protein standards and protein fractions re-analyzed on SDS–PAGE under reducing conditions.

all three fusion proteins, indicating that recombinant CTB fusion proteins with either C- or N-terminal or dual extensions have the ability to pentamerize. Integration of the peak areas on the chromatograms allows to estimate the ratio between potentially pentameric and monomeric forms of the different fusion proteins. It appears that C-terminal fusions to CTB are preferable to N-terminal fusions, since the CTB-BB fusion protein shows the highest degree of pentamerization (predominant pentamer peak), while BB-CTB shows a more equal distribution between the pentamer and monomer peaks (Fig. 2). However, the dual fusion to CTB, resulting in the BB-CTB-BB fusion protein, seems to have somewhat affected the ability to form pentamers.

To further analyze and characterize the potentially pentameric forms of the three fusion proteins, preparative size-exclusion chromatography was used to collect the fractions expected to contain the pentamers. These fractions where subjected to SDS–PAGE under non-reducing conditions, and Western blotting, using either polyclonal CTB-reactive rabbit serum or a monoclonal antibody (LT39) which is specific for CTB-pentamers (González et al., 1993). Since both immunoblots showed almost identical patterns, we chose to include only the results from the blot obtained when using the monoclonal anti-

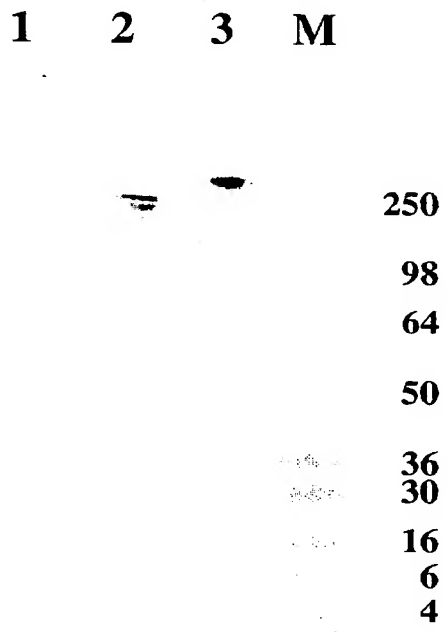

Fig. 3. Immunoblot analysis of the CTB fusion proteins. Samples collected from the fractions which were suggested to contain pentameric forms of the three fusion proteins (peak I, Fig. 2) were separated on a 4–20% gradient gel and tested for reactivity in an immunoblotting experiment to a monoclonal antibody LT39 specific for CTB-pentamers (González et al., 1993). Lane 1, CTB-BB; lane 2, BB-CTB; lane 3, BB-CTB-BB; lane M, pre-stained marker proteins with molecular masses in kilodaltons.

body (Fig. 3). Furthermore, collected peak II-fractions were not stained by the monoclonal antibody in immunoblotting (data not shown) suggesting that these fractions did not contain pentameric material. First of all, the results demonstrate that the peak I-fractions collected from the size-exlusion chromatography indeed contained material of sizes compatible with pentamers, in agreement with the assumptions made from Fig. 2. Secondly, although the CTB-BB material seems to be partly degraded (Fig. 3, lane 1), the reactivity in the blotting using the pentamer-specific monoclonal antibody (Fig. 3), suggests that pentameric fusion proteins indeed were formed from all three CTB fusion proteins.

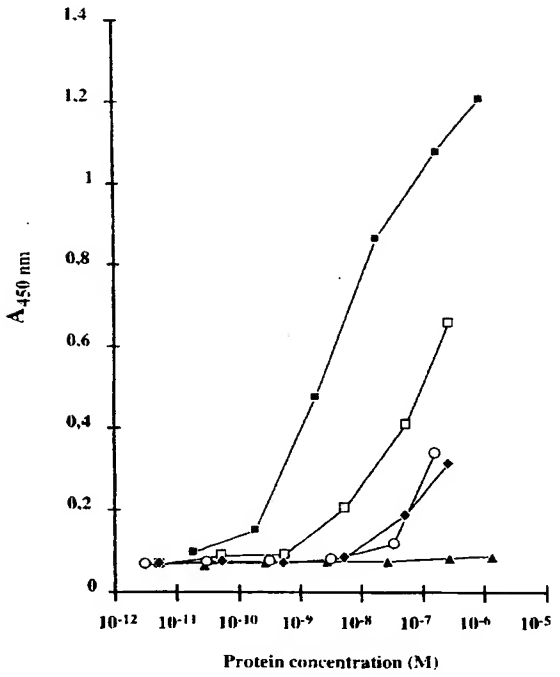

Fig. 4. GM1 binding analysis of the CTB fusion proteins. Abilities of the pentamer fractions of the CTB fusion proteins to bind to the GM1 ganglioside was measured by ELISA. Microtiter plates, precoated with GM1, were treated with samples collected from the size-exclusion chromatography fractions which were suggested to contain pentameric forms of the fusion proteins (peak I, Fig. 2). The samples were diluted to various molar concentrations of the fusion proteins: CTB-BB (□), BB-CTB (◆), and BB-CTB-BB (○). Native CTB (■) was included as positive control, and a fusion protein BB-M3 (▲) was used as negative control. To detect bound proteins, wells were incubated with anti-CTB polyclonal antisera and HRP-labelled secondary antibodies, followed by the addition of substrate, and the absorbance at 450 nm was finally monitored. Data are presented as mean values of triplicate determinations.

3.4. GM1 binding

Since true functionality of CTB pentamers is correlated with the ability to bind the GM1 ganglioside, an ELISA was set up in order to determine the GM1 binding capacity of the pentamers formed by the three different CTB fusion proteins. GM1 was used as coating reagent in the microtiter wells and pentamers of the CTB fusion proteins, collected by size-exclusion chromatography, were analyzed in serial dilutions, to generate binding curves. Commercial CTB and an affinity-purified irrelevant BB fusion protein, BB-M3 (Ståhl et al., 1990), were used as positive and negative controls, respectively. Complete binding curves could not be obtained since saturation levels (plateau values) could not be reached, but the binding curves nevertheless demonstrate that all three chimeric CTB pentamers bound GM1 (Fig. 4), although not as strongly as commercial CTB. The C-terminal CTB fusion protein, CTB-BB, showed good GM1 binding, while the N-terminal fusion (BB-CTB) and the dual fusion (BB-CTB-BB) showed significant decrease in GM1-binding capacity. All samples were analyzed in triplicates.

4. Discussion

Three different CTB fusion proteins were produced in *E. coli*, affinity purified on HSA–Sepharose, and compared for there ability to pentamerize and bind the cell receptor for CTB, the GM1 ganglioside. It was found that all three fusion proteins could form pentamers, although the C-terminal fusion was found to be the most efficient. The pentamer fractions formed from the three fusions were recovered by size-exclusion chromatography and it was demonstrated that they reacted in immunoblotting with a monoclonal antibody specific for CTB pentamers. Furthermore, the pentamers formed from the three CTB fusion proteins were all able to bind GM1, although the C-terminal fusion (CTB-BB) with a higher reactivity than the N-terminal and dual

fusions, for which the binding was just on the border of being significant. This poorer GM1-binding capacity of the pentamers assembled from the N-terminal and dual CTB fusions is most likely due to sterical hindrance, since recent structural data (Zhang et al., 1995a,b) and mutational analysis (Bäckström et al., 1997) suggest that the N-terminus of CTB is in closer proximity to the GM1 binding pocket. Taken together, this shows that CTB, as a carrier protein, is rather permissive to gene fusions, since both C-terminal, N-terminal as well as dual CTB fusion proteins, at least in our system, are able to form pentamers and bind GM1. Nevertheless, the differential behavior in terms of pentamerization efficiency and GM1 binding suggests that new CTB fusion proteins should be carefully characterized prior to immunization experiments. In our model system, C-terminal fusions seem comparatively more attractive, although the N-terminal fusion shows a somewhat better proteolytic stability (Fig. 3). We believe that the reason why the described fusion proteins have proven to be able to form pentamers and bind GM1, is that the fusion partner BB is able to fold separately and efficiently. Fusions to structurally distinct domains are more likely to fold independently and not to interfere with the activity of the fused target protein (Nygren et al., 1994), as also demonstrated by Jagusztyn-Krynicka et al. (1993) for LTB fusions.

Monomeric forms of CTB and CTB fusion proteins have been previously reported not to bind GM1 (Hardy et al., 1988). We tried to confirm this conclusion by disrupting pentamers either by changes in temperature or pH. However, we found it impossible to find conditions where pentamers were reversibly disrupted and remaining in a monomeric form long enough time for any assay to be performed. For example, CTB pentamers can be disrupted at pH 3, without causing irreversible denaturation, but upon neutralization which is needed for the GM1 ELISA, pentamers form with high efficiency, making it impossible to analyze monomers (data not shown).

Also other strategies have been evaluated in order to utilize the immunopotentiating properties of CTB. Recently, chemically synthesized peptides comprising a 26 amino acid epitope from CTB and a 14 amino acid from the G protein of RSV, were used to immunize mice (Delmas and Partidos, 1996). The synthetic peptides could inhibit the binding of CTB to GM1 in vitro, and elicited significant immune responses after intranasal delivery (Delmas and Partidos, 1996). An elegant example of genetic engineering was reported by Hajishengallis et al. (1995), where the toxic A1 subunit of CT was replaced by a 42 kDa streptococcal adhesin (SBR). An SBR-A2 fusion was coexpressed with CTB in *E. coli*, and recovered SBR-A2:CTB_5 complexes retained their GM1 binding property and elicited significant immune responses (Hajishengallis et al., 1995).

In conclusion, the present results demonstrate, that at least in the described system, CTB can indeed be used as a carrier for large C- or N-terminal fusions while keeping the ability to form pentamers, and to bind to the GM1 ganglioside. CTB should thus be suitable for targeting, not only shorter peptides but also larger antigens, to the mucosal epithelium. This ability might even be of value in increasing the immunogenicity of live bacterial vaccine vehicles via surface display of CTB, since it has been shown that CTB can be produced in a functional form on the surface of recombinant staphylococci intended for vaccination purposes (Liljeqvist et al., 1997). Furthermore, since BB has been demonstrated to exhibit carrier-related properties which have proven beneficial for the delivery of subunit vaccines (Sjölander et al., 1997; Power et al., 1997), it would be of interest to evaluate whether the fusion proteins described in this study show increased immunogenicity as compared to non-fused CTB. This report also suggests rational strategies for characterization of CTB fusion proteins used in the context of vaccine development.

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